UNIVERSITY *of* NORTH CAROLINA WILMINGTON

October 31, 2016

Defense Technical Information Center
8725 John J. Kingman Road Ste 0944
Fort Belvoir, Virginia 22060-6218

Grant #: N00014-12-1-0442

To Whom It May Concern:

The University of North Carolina at Wilmington is pleased to submit the attached final program report with completed SF298 form for the funded project, "Nitrogen Solubility in Adipose Tissues of Diving Animals: Implications for Human Divers and for Modeling Diving Physiology," Dr. Heather Koopman, lead Principal Investigator. We thank Office of Naval Research for the support of this successful project and hope to work together on future endeavors.

Please do not hesitate to contact me at powellp@uncw.edu or 910-962-3167 if I may provide further information.

Sincerely,

Panda Powell

Panda Powell
Director
Office of Sponsored Programs and Research Compliance

Cc: Dr. Heather Koopman
file 572480

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-11-2016	Final report	April 1 2012-Aug 1 2016

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Nitrogen Solubility in Adipose Tissues of Diving Animals: Implications for Human Divers and for Modeling Diving Physiology	
	5b. GRANT NUMBER N00014-12-1-0442
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Heather Koopman, Andrew Westgate, Molly Gabler	
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Biology & Marine Biology, UNCW 601 S. College Road Wilmington, NC 28403	572480

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
Office of Naval Research 875 North Randolph Street Arlington, VA 22203-1995	ONR
	11. SPONSOR/MONITOR'S REPORT NUMBER(S) 572480 - FINAL

12. DISTRIBUTION/AVAILABILITY STATEMENT

Report is public availability.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There is currently little information on how N2 solubility in adipose tissue varies with its lipid composition, and how it varies across species. We proposed to generate these much-needed empirical data by comparing nitrogen solubility in the adipose of marine mammals, seabirds, and turtles, and in mammals used as models for diving physiology, as well as human tissue, in conjunction with the degree of microvascularity (exchange surface). We found considerable variation across species/animal groups in nitrogen solubility, lipid composition, and microvascular components. While for most species (those lacking waxes and short/branched chain fatty acids/alcohols) it may be appropriate to use a "standard" value for nitrogen solubility around our overall mean of 0.064, this does not apply to all animal groups (e.g. seabirds, had very low values ~ 0.055). Also, nitrogen solubility values for the human and pig samples were very similar, making it tempting to use a value of ~ 0.064 – 0.065 for any diving physiology models including adipose as a compartment in terrestrial mammals. However we advise caution, given the small sample size and the variability in solubility values among the marine species. Therefore it is important to obtain representative nitrogen solubility values for taxonomic groups not yet examined.

15. SUBJECT TERMS

nitrogen solubility, adipose, diving physiology, vascularity

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Panda Powell
U	U	U	U	1	19b. TELEPHONE NUMBER *(Include area code)* 910-962-3167

Final Report

I. Grant Information

PI: Dr. Heather Koopman
Institution: Biology & Marine Biology, University of North Carolina Wilmington
Award #: N000141210442
Title: Nitrogen Solubility in Adipose Tissues of Diving Animals: Implications for Human Divers and for Modeling Diving Physiology

II. Scientific and Technical Objectives

Background:

Understanding the solubility of nitrogen gas in tissues is a critical element in studies of diving physiology, especially for air-breathing tetrapods. Adipose tissue is of particular concern, because N_2 is 5 times more soluble in lipid than water and thus at any blood/fat interface, N_2 will move into fat (Ikels 1964; Gerth 1985). Adipose tissue is also recognized as playing an important role in DCS (decompression sickness) risk in diving mammals (Dembert et al. 1984; Carturan et al. 2002). The amount of fat within an individual's body can affect the occurrence and severity of DCS: there is considerable evidence that obesity is linked to higher rates of DCS in humans and other animals (Rattner et al. 1979; Dembert et al. 1984; Douglas 1985; Lam and Yau 1989; Carturan et al. 2002). However, beyond some preliminary data generated by our lab (see below), there is currently very little information on how N_2 solubility in adipose tissue might vary with the specific lipid composition of that tissue, and how it might vary across species. Such data are of extreme importance when modeling human diving physiology, as parameters employed in such models are usually based on assumptions from generalized tissues without more specific empirical data. We proposed to generate this much-needed empirical data, and also to examine several aspects of the evolution of diving physiology in air-breathing animals by comparing nitrogen solubility in the adipose of marine mammals, seabirds, and turtles, and (with direct application to Naval Operations) adipose tissues of mammals routinely used as models for diving physiology (pigs and sheep) as well as human tissue.

Through a previous grant from ONR (N00014-06-0308), our lab spent several years developing a new method for measuring nitrogen solubility in oils by incubating oils and pure nitrogen gas in headspace vials inside a sealed argon environment and quantifying N_2 liberated from the oils post-incubation via gas chromatography (GC). The apparatus was tested and went through QA/QC procedures using olive oil, as there are published data for this material. Our olive oil values fell right within the range of published data for this material so we are confident about our methodology. We then carried out some preliminary analyses of N_2 solubility in oils extracted from blubber of toothed whales; these data showed that whale tissues exhibit nitrogen solubility values that are variable across species, are mostly higher than olive oil, and are linked to variation in lipid composition (Koopman and Westgate 2012). Specifically, lipids with higher wax ester content (as found in sperm and beaked whales; one fatty acid linked to one fatty alcohol) showed significantly higher N_2 solubility values (up to 40% higher) than lipids composed entirely of triacylglycerols, the typical mammalian storage fat composed of three fatty acids linked to a glycerol backbone. However, lipid class explained only 52% of the variation in nitrogen solubility in the species we tested, suggesting that a more detailed examination of lipid composition, across a wider range of diving animals, was warranted. We aimed to cover diving seabirds, turtles, seals and non-baleen whales.

We also wanted to include some human tissue in our new study. The N_2 solubility work that does exist for humans has been mostly limited to the blood or brain (e.g. Farhi et al. 1963; review by Weathersby and Homer 1980; Yamaguchi et al. 1993; review by Langø et al. 1996). There are only a few mentions in the literature of N_2 solubility coefficients for human adipose: the review by Steward et al. (1973) gives a value of 0.07 for human fat "extract", and Ikels (1964) presents 0.061; these data were collected using completely different methods and under different conditions. We could not find any data on nitrogen gas solubility in human adipose tissue in the primary literature that have been published more recently than 1973, almost four decades ago. Given the role of nitrogen gas during diving, and its relationship to health and risk of DCS for those undergoing repetitive dives, it would seem not only appropriate but absolutely critical for our understanding of diving physiology to update these values, and to make these determinations on a greater sample size than previous studies have (given the variation in the above published values), and with the same set-up that we will use to investigate solubility in other species to minimize any instrumentation bias.

We also proposed to extend the study to compare nitrogen solubility in adipose at different temperatures, because diving animals and humans may experience a range of water temperatures when diving to different depths or in different locations, and even though mammalian and avian body core temperature is regulated to between 37-40 °C, the peripheral regions of the body will experience temperatures along a gradient between core and ambient.

Coupled with new knowledge of solubility in adipose tissue was the need to quantify the potential for exchange between the circulatory system and adipocytes at the microvascular level. In order for lipid composition to play a role in N_2 gas dynamics, a blood/adipose interface must exist. Typically, adipose tissue is perfused by networks of microvasculature: capillaries, microarterioles and microvenules (Gersh and Still, 1945; Herd et al., 1968; Hausman and Wright, 1996; Crandall et al., 1997) for delivery and exchange of gases, nutrients and wastes. In terrestrial animals, adipose tissue is considered to be "highly vascularized" (Gersh and Still, 1945; Herd et al., 1968; Hausman and Wright, 1996; Crandall et al., 1997), yet despite this generalization, there is remarkably little quantitative information on microvasculature in adipose tissues – even for humans. In our first ONR study, we quantified microvasculature in several layers of the blubber of bottlenose dolphins (shallow divers), pygmy sperm whales (intermediate divers), and beaked whales (deep divers) in comparison with adipose from a standard terrestrial model (pigs) and found significant variation across these species. The shallow-diving dolphins had far greater microvascularity than the deeper diving species and the pigs, suggesting variation in this nitrogen dynamics parameter across species as well. We wanted to capture and quantify this variation by examining the adipose tissue of a diverse group of diving tetrapods, and terrestrial species used in diving models.

Finally, in addition to our primary goal of comparing nitrogen solubility in adipose across many diverse vertebrate air breathers, we also wanted to continue a line of research stemming from an observation made during the previous ONR grant, when we examined different adipose tissues from the same individual. Toothed whales echolocate and have evolved specialized fat bodies in their foreheads and lower jaws to transmit and receive sound, respectively (Norris 1968). These fat bodies are composed of endogenous lipids that are not found synthesized or deposited by any other mammals (e.g. short, branched chain fatty acids, some of which are toxic to most mammals – Wretland 1957; Tanaka et al. 1966; Litchfield et al., 1975; Koopman et al. 2003, 2006) and are assumed to be crucial for the echolocation process (e.g. Blomberg and Lindholm 1976; Morris 1986). As the composition of the "acoustic fats" is so unusual, and is very different from that of the blubber, we carried out a preliminary comparison of nitrogen solubility in the blubber and lower jaw fats of a Risso's dolphin in our first ONR grant to evaluate whether there were any possible differences between these two types of adipose tissue. The acoustic fats of Risso's dolphins are rich in isovaleric acid (*i*-5:0) and isopentadecanoic acid (i-15:0), molecules derived from the breakdown of leucine (see Koopman et al. 2003; Koopman unpublished data). The acoustic fat (jaw fat) sample had a 16% higher relative nitrogen solubility than the blubber sample from the same animal (Koopman and Westgate 2012), suggesting that there may be differences in nitrogen gas dynamics between blubber and the specialized acoustic fats within a single animal. In addition to being an interesting basic research question about how the acoustic fats behave physiologically, the comparison of N_2 solubility in blubber vs. melon and jaw fats may provide insight into the sensitivity of these tissues to formation of emboli under different diving conditions.

Our study had four main objectives:
1) compare solubility of nitrogen in adipose tissues across a wide array of air-breathing animal species: a) those that are adapted for diving: marine mammals (seals, sea lions, whales, dolphins), seabirds, and turtles; b) terrestrial species used as models in diving physiology: pigs, sheep; c) humans. We also plan to compare the acoustic (echolocation) tissues of toothed whales with that of their blubber to determine if there are systematic differences in nitrogen dynamics across adipose compartments within an animal.
2) determine the effect of temperature on solubility in tissues above in a range of biologically relevant temperatures (0 to 37 °C).
3) determine patterns of vascular density and morphology in tissues above, compare across species and evaluate relationship to nitrogen solubility.
4) determine lipid profiles (content, lipid classes, fatty acid composition) of adipose tissues of all species examined above and evaluate whether lipid types and nitrogen solubility are linked in other animals as they appear to be in toothed whales.

III. Approach
Prior to this project, there was currently very little information on how N_2 solubility in adipose tissue might vary with the specific lipid composition of that tissue, and how it might vary across species beyond some preliminary data generated by our lab (Koopman and Westgate 2012). We proposed to generate these much-needed empirical data, and also to examine several aspects of the evolution of diving physiology in air-breathing animals by comparing nitrogen solubility in the adipose of marine mammals, seabirds, and turtles, and (with direct application to Naval Operations) adipose tissues of mammals

routinely used as models for diving physiology (pigs and sheep) as well as human tissue. Thus we hoped to link lipid composition with nitrogen solubility, and also quantify microvasculature in these adipose tissues to evaluate the potential for nitrogen loading in adipose depots from different animals.

Brief methods:

1. Identification of lipid content and composition: lipids were extracted using a conventional Folch extraction of chloroform/methanol (Folch et al. 1957) with some modifications (Koopman 2007). Lipid classes were identified and quantified using thin-layer chromatography flame ionization detection on an Iatroscan (Koopman 2007). Fatty acids and alcohols were identified and quantified on a Varian 3800 gas chromatograph using Galaxie software, with peak identification based on standard reference oils and confirmed on GC/MS.
2. Nitrogen solubility was measured using our redesigned apparatus in a multistep process that involved bubbling pure N_2 through extracted lipids, and incubating these in headspace vials to measure the amount of nitrogen given off, all in an argon environment to minimize environmental contamination. Components were jacketed in warming tubes controlled by a precision Fisher Scientific water bath.
3. Microvasculature has been quantified in adipose tissues using previously developed methods (see McClelland et al. 2012).

IV. Concise Achievements

Over the course of this project (2012-2016) we have accomplished the following:

- 52 samples, representing 18 species, analyzed for nitrogen solubility
- 103 samples processed for lipid content and composition
- 89 samples analyzed for microvascularity
- 12 presentations at regional/international scientific conferences (10 of them by students, both graduate and undergraduate)
- 1 paper published in Journal of Experimental Biology (and picked up by the media; see X.G. below); 3 additional papers in preparation.
- Two graduate students (1 Ph.D. student still in progress; 1 M.Sc. student who graduated in 2014) were trained on nitrogen solubility and lipid extraction techniques; both contributed directly to the dataset of this project. An additional M.Sc. Student (Yanes) and two undergraduates (Ernst and Pelletier) carried out research that was not directly related to the objectives of this project, but which used the same samples and many of the same techniques to address slightly different questions about marine mammal lipids. See X.E. for details on the presentations stemming from this work.

V. Expanded Accomplishments

Nitrogen solubility

Across all samples measured, nitrogen solubility in oils extracted from adipose tissue ranged from 0.051 (Adelie penguin) to 0.105 (acoustic fat of short-finned pilot whale) Ostwalds. The average for all 52 samples was 0.073 ± 0.013 (Table 1). Although this overall mean is very close to the generalized "fat" nitrogen solubility value of 0.07 typically used in the literature and in diving physiology calculations, it should be noted that the variation from species to species was over 100% (i.e. doubling from the Adelie penguin to the pilot whale). In fact, there was significant variation even within a species if different fat depots were examined. For example, if one considers only the toothed whales, the nitrogen solubility values for the acoustic fats were significantly higher ($P<0.0001$; by 13-27%) than those for blubber, and even within the acoustic fats we noted ~13% higher solubility in the extramandibular fat bodies (covering the mandible, under the blubber) than the intramandibular fat bodies (within the mandible, directly attached to the ear) (Table 2).

We were very excited that after much bureaucratic, administrative and logistical difficulty, we have obtained the first data on nitrogen solubility in *human adipose* in over 40 years! Human tissue has an unremarkable nitrogen solubility (0.065 ml $\pm$ 0.004 N_2/ml oil; multiple runs on one sample) that is similar to that of several of the other species we have examined (grey seal, sea lion, and the pig; Table 1). Our values fall between those published 4-5 decades ago for human oils using different methods (0.064 and 0.07; Ikels 1964 and Steward et al. 1973). Once we analyze our fatty acid data for the human sample we will be able to see how it compared with our marine species. These data will appear in a future manuscript.

We had difficulty measuring nitrogen solubility in the other terrestrial species (goat, cow, sheep) because these lipids are solid at mammalian body temperature (37ºC) and thus we could not bubble nitrogen through an oil phase in our apparatus.

Table 1. Nitrogen solubility, lipid content and wax ester content of biological oils extracted from animal tissues that are storage depots (adipose, blubber). Values are means for the species ± standard deviation. Sample sizes are given in parentheses.

Species	Common Name	N_2 solubility (Ostwalds)	Lipid content (wt%)	Wax ester content (wt% of total lipids)
Aptenodytes forsteri	Emperor penguin	0.054 (1)	50.5 (1)	0.0 (1)
Phygoscelis adeliae	Adelie penguin	0.055 ± 0.005 (2)	62.3 ± 3.2 (2)	0.0 (2)
Somateria mollisima	Eider duck	0.055 (1)	73.2 ± 7.5 (3)	0.0 (3)
Dermochelys coriacea	Leatherback turtle	0.061 ± 0.002 (3)	55.0 ± 17.7 (4)	0.0 (5)
Caretta caretta	Loggerhead turtle	-	71.2 ± 10.5 (2)	0.0 (2)
Chelonia mydas	Green turtle	-	37.6 (1)	0.0 (1)
Halicheorus grypus	Grey seal	0.064 (1)	66.4 (1)	0.0 (1)
Phoca vitulina	Harbour seal	-	83.7 ± 3.8 (6)	0.0 (6)
Pagophilus groenlandicus	Harp seal	-	76.2 ± 5.5 (2)	0.0 (2)
Zalophus californianus	California seal lion	0.062 (1)	69.6 ± 6.0 (4)	0.0 (4)
Balaenoptera acutorostrata	minke whale	0.061 ± 0.003 (3)	42.9 ± 22.7 (9)	0.0 (9)
Balaenoptera borealis	sei whale	0.062 (1)	52.3 (1)	0.0 (1)
Balaenoptera physalus	fin whale	-	48.9 ± 24.2 (3)	0.0 (2)
Stenella frontalis	Atlantic spotted dolphin	0.062 ± 0.007 (3)	53.1 ± 3.0 (3)	0.0 (3)
Globicephala macrorhyncus	Short-finned pilot whale	0.066 ± 0.006 (3)	57.8 ± 4.1 (4)	0.0 (4)
Kogia breviceps	Pygmy sperm whale	0.075 ± 0.001 (3)	44.9 ± 8.3 (3)	94.3 ± 1.1 (3)
Physeter macrocephalus	Sperm whale	0.072 (1)	40.4 (1)	98.5 (1)
Mesoplodon densirostris	Blainville's beaked whale	0.072 (1)	64.7 (1)	98.9 (1)
Mesoplodon europaeus	Gervais' beaked whale	0.073 (1)	72.8 (1)	81.5 (1)
Hippopotamus	hippopotamus	-	82.5	0.0

amphibius			(1)	(1)
Bos taurus	cow	-	76.2 (1)	0.0 (1)
Capra aegagrus hircus	goat	-	79.8 (1)	0.0 (1)
Ovis aries	sheep	-	82.6 (1)	0.0 (1)
Sus scrofa	pig	0.066 (1)	75.1 ± 6.5 (4)	0.0 (4)
Homo sapiens	human	0.065 (1)	63.5 ± 1.8 (2)	0.0 (2)
Overall		0.064 ± 18.5 (27)	61.0 ± 18.5 (64)	8.6 ± 27.4 (65)

Table 2. Nitrogen solubility (Ostwalds) and wax ester content (wt% of total lipids) of oils extracted from blubber and acoustic fats of toothed whales (Odontocetes). Values are means for the species ± standard deviation. Sample sizes are given in parentheses.

Species	N_2 solubility			Wax ester content		
	blubber	EMFB	IMFB	blubber	EMFB	IMFB
Atlantic spotted dolphin	0.062 ± .007 (3)	.086 ± 0.003 (3)	0.076 ± 0.007 (3)	0.0 (3)	21.4 ± 6.7 (3)	7.9 ± 2.2 (3)
Short-finned pilot whale	0.066 ± 0.006 (3)	0.101 ± 0.004 (3)	0.093 ± 0.004 (3)	0.0 (3)	34.3 ± 18.1 (4)	13.9 ± 4.6 (4)
Risso's dolphin (*Grampus griseus*)	-	-	-	-	30.7 (1)	3.5 (1)
Narwhal (*Monodon monoceros*)	-	0.082 (1)	-	-	0.8 (1)	-
Pygmy sperm whale	0.075 ± 0.001 (3)	0.085 ± 0.001 (3)	0.066 ± 0.003 (3)	94.3 ± 1.1 (3)	62.8 ± 26.5 (5)	19.9 ± 11.9 (5)
Dwarf sperm whale (*K. sima*)	-	-	-	-	46.1 ± 2.8 (2)	27.3 ± 13.7 (2)
Sperm whale	0.072 (1)	0.081 (1)	0.075 (1)	98.5 (1)	98.7 (1)	75.6 (1)
Sowerby's beaked whale (*M. bidens*)	-	-	-	-	54.1 (1)	50.3 (1)
Blainville's beaked whale	0.072 (1)	0.081 (1)	0.079 (1)	98.9 (1)	46.1 (1)	74.7 (1)
Gervais' beaked whale	0.073 (1)	0.083 (1)	0.076 (1)	81.5 (1)	41.7 ± 20.2 (2)	36.5 ± 3.6 (2)
True's beaked whale (*M. mirus*)	-	-	-	-	81.8 (1)	25.4 (1)
Cuvier's beaked whale (*Ziphius cavirostris*)	-	-	-	-	18.4 (1)	16.0 (1)

It is also clear that overall the toothed whales (Odontocetes) have higher nitrogen solubility than other categories of species. We divided the samples into the following categories: terrestrial species, turtles, seabirds, pinnipeds (seals and sea lions), whales (cetacean species not included in Table 2), and toothed whales. There were significant differences across these groups in nitrogen solubility (ANOVA; $P<0.0001$), even if the toothed whale acoustic samples are excluded (ANOVA; $P = 0.002$; Figure 1). The seabird samples have the lowest solubility and the toothed whale blubber the highest; all of the other groups have fairly similar values that are not significantly different.

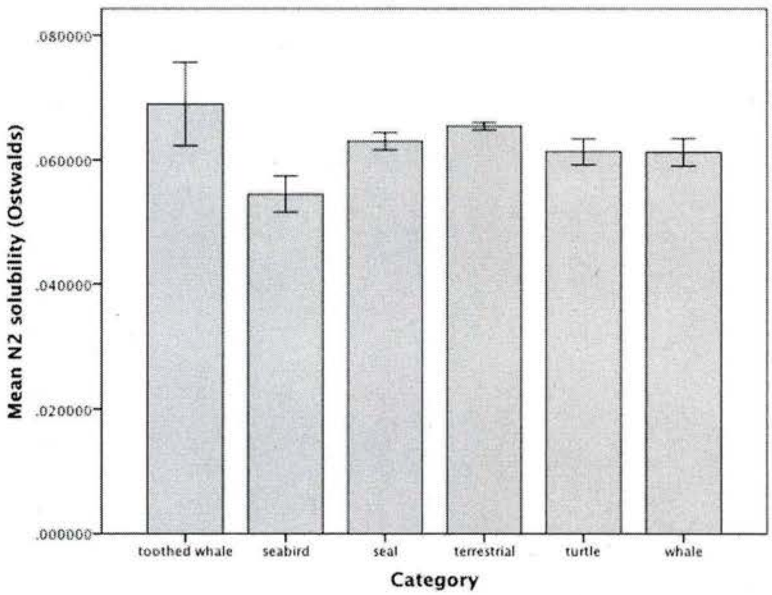

Figure 1. Mean nitrogen solubility (Ostwald units ± standard deviation) for storage depots (adipose, blubber) in animals of different taxonomic groups.

As with our first study (Koopman and Westgate 2012), overall there was a significant relationship between wax ester content and nitrogen solubility (P=0.002; Figure 2). However, when all samples are considered together, WE content only explains 15% of the variation in nitrogen solubility ($R^2 = 0.155$). This is largely because most samples do not contain any waxes (Table 1); this lipid class is found exclusively in the toothed whales in this study (Table 2).

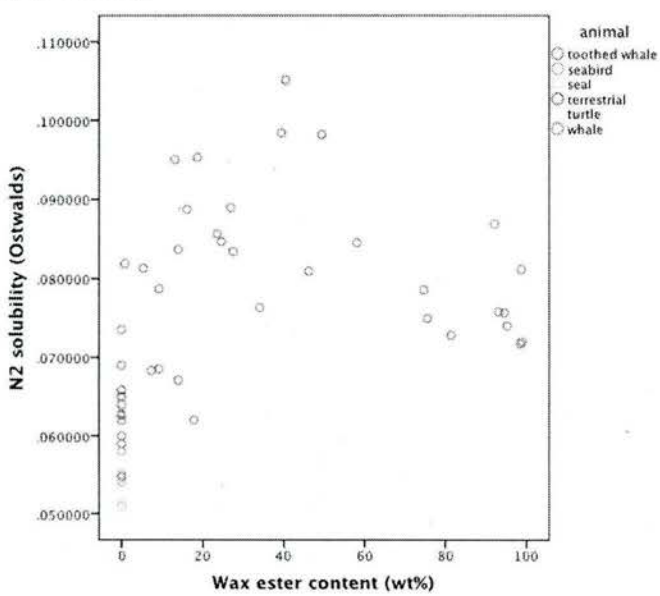

Figure 2. Relationship between wax ester content of biological oils and nitrogen solubility.

If only the toothed whales are examined, the relationship becomes more complicated as the acoustic fats behave differently than blubber in terms of increasing solubility values with increasing wax content (Figure 3).

Unraveling this relationship was the main focus of Gina Lonati's M.Sc. thesis and the main result of the publication that came from her work (Lonati et al. 2015). We found that the influence of waxes on solubility was more pronounced in mandibular fats with higher concentrations of shorter, branched fatty acids/alcohols. That is, in species with greater fractions of their triacylglycerols and wax esters that are composed of short and branched building blocks, nitrogen solubility was higher.

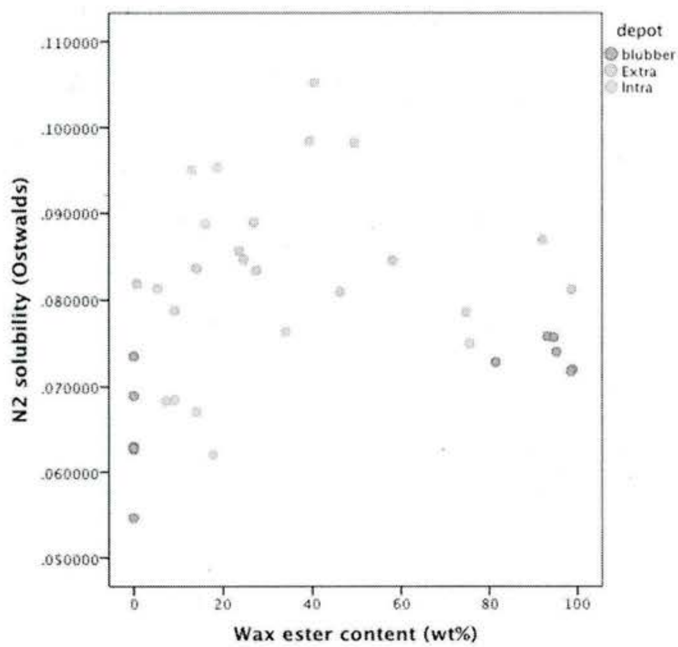

Figure 3. Relationship between wax ester content and nitrogen solubility in oils extracted from toothed whale tissues, separated by tissue type (blubber vs. mandibular acoustic fats [Extra- and Intra-mandibular fat bodies]).

When Gina's nitrogen solubility data were plotted against wax content, but coded by species and tissue type, several patterns emerged (Figure 4).

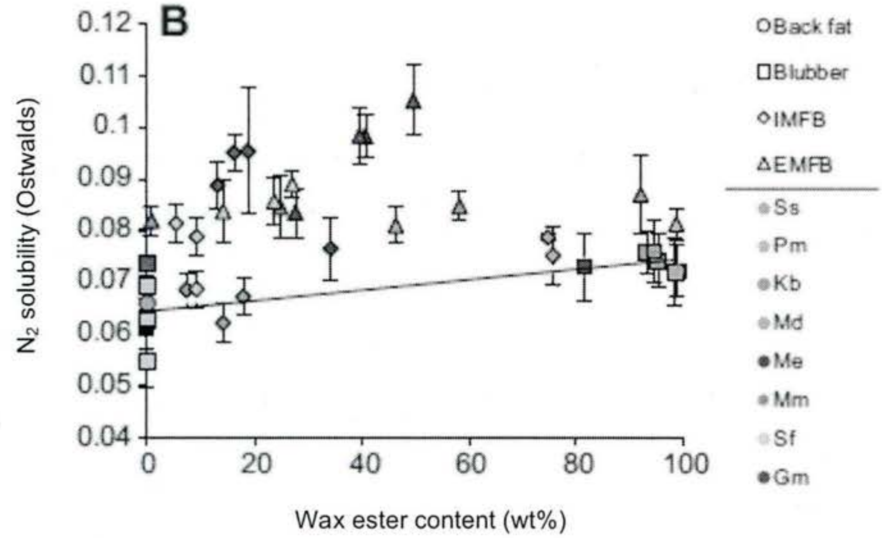

Figure 4. Relationship between wax ester content and nitrogen solubility in toothed whales, coded by tissue type and species. IMFB = intramandibular fat body, EMFB = extramandibular fat body (both acoustic tissues), Ss = pig, Pm = sperm whale, Kb = pygmy sperm whale, Md = Blainville's beaked whale, Me = Gervais' beaked whale, Mm = narwhal, Sf = spotted dolphin, Gm = pilot whale. This is Figure 4B from Lonati et al. (2015), Journal of Experimental Biology.

First, there was a significant relationship between wax content and nitrogen solubility only for blubber (not the acoustic tissues) ($P<0.01$, $y=0.0001x+0.064$, $R^2=0.54$). Second, the rate of increase in nitrogen solubility in the acoustic tissues as a function of wax ester content varied across species. For example, for a given wax content, pilot whales (dark blue) exhibited higher solubility values than the other species. As we investigated further it became clear that the individual components comprising the waxes (and the triacylgylcerols) varied across species and had an influence on solubility (Figure 5).

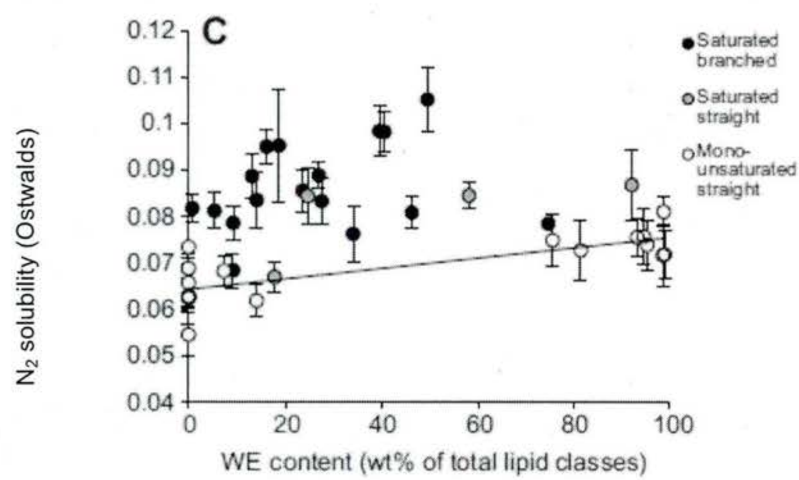

Figure 5. Relationship between wax ester content and nitrogen solubility in toothed whales, coded by major fatty acid/alcohol components – these are the same data as Figure

4, but coded by lipid composition rather than species. This is Figure 4C from Lonati et al. (2015), Journal of Experimental Biology.

In marine species, most fatty acids in triacylglycerols and waxes are long (16+ carbons), straight chains with one double bond between adjoining carbons (monounsaturated) or multiple double bonds (polyunsaturated). Most of the fatty alcohols are saturated (no double bonds; all carbons "saturated" with hydrogens) with a few monounsaturated components. Infrequently are fatty acids saturated, and very rarely are they branched (essentially a branch linking 3 carbons at the methyl terminus) – except in the case of the endogenous acoustic fats of toothed whales (see *Background* section). These branches confer differing physical and acoustic properties to lipids (e.g. reducing the speed at which sound travels through them) and they also appear to lead to an increase in nitrogen solubility. An ANOVA and Tukey's post hoc test demonstrated that samples dominated by saturated branched chains had a significantly higher average N_2 solubility than those dominated by monounsaturated straight chains ($P<0.01$). Finally, the branched components in the acoustic fats are often shorter than normal fatty acids (5-12 carbons). Shorter lipids also have higher nitrogen solubility (Figure 6).

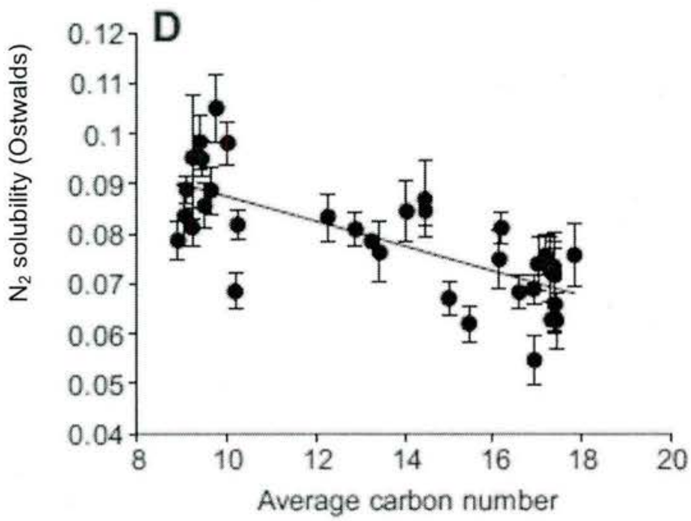

Figure 6. Relationship between average carbon number (measure of the length/size of lipid molecules) and nitrogen solubility in toothed whales. This is Figure 4D from Lonati et al. (2015), Journal of Experimental Biology. The linear regression was significant ($P<0.01$, $y=-0.002x+0.112$, $R^2=0.54$), such that N_2 solubility was negatively correlated with average carbon number.

The major conclusions of Lonati et al. (2015) were: "This study is the first to link N2 solubility with the fatty acid and fatty alcohol composition of lipids in marine mammals. It specifically demonstrates that N_2 solubility in odontocete blubber and mandibular fats differs and is influenced by the unique lipids these animals synthesize. As composition varies with both phylogeny and fat depot, different species may experience varying N_2 loads in different fatty tissues." The significance of this work was that "Larger quantities of shorter, saturated branched-chain fatty acids and fatty alcohols, combined with increased WE content, appear to increase N_2 solubility above the value typically used for modeling gas dynamics in diving whales", with the end result being that pilot whales and deep-diving beaked whales may experience greater nitrogen loading, and perhaps risk of DCS, than other species, just by the nature of the lipids they synthesize for echolocation.

For the other species (seals, turtles, seabirds, terrestrial animals, humans) the fatty acid data have been collected but not yet analyzed. However we know that none of these species have any waxes in their adipose depots, and given that their nitrogen solubilities are fairly similar we do not expect there to be large influences of fatty acid composition on nitrogen solubility – the only exception to this generalization could be the seabirds, which had significantly lower solubility than the other animal groups. Those data will be forthcoming but we do not have them at the time of writing this report.

Thermal effects on nitrogen solubility

This was the aspect of the project that we did not complete to the degree that we had hoped. We carried out our initial temperature experiments with olive oil (this was three months' worth of work alone – 92 nitrogen solubility runs). These data suggest that nitrogen solubility decreases with decreasing temperature; Ostwalds are given below (mL N_2/mL oil).

At 37°C = 0.063 – 0.065 ± 0.003

At 34°C = 0.0614 ± 0.004

At 25°C = 0.0466 ± 0.003

Our preliminary experiments with adipose tissue from leatherback turtles also suggest that N_2 solubility decreases with decreasing temperature, at least in this species; Ostwalds are given below (mL N_2/mL oil).

At 37°C = 0.064

At 36°C = 0.063

At 34°C = 0.061

We also have some (very) preliminary data from beaked whale acoustic fat samples that suggest a ~10°C temperature decrease may result in a 20-30% decrease in nitrogen solubility. Further work is needed here.

Microvascularity

The density of microvessels (capillaries, microarterioles, microvenuoles) varied considerably (16-fold) across all samples examined, from 0.51 (beaked whale) to 8.11 (eider duck) (Tables 3 and 4).

Table 3. Microvascularity (% of area of tissue occupied by microvessels) and degree of branching in animal adipose tissues that are storage depots (adipose, blubber). Values are means for the species ± standard deviation. Sample sizes are given in parentheses.

Species	Common Name	Microvascularity	Microvascular Branching
Phygoscelis adeliae	Adelie penguin	5.07 ± 0.48 (2)	2.1 ± 0.1 (2)
Somateria mollisima	Eider duck	6.60 ± 2.14 (2)	2.6 ± 0.2 (2)
Dermochelys coriacea	Leatherback turtle	5.70 ± 1.21 (4)	2.4 ± 0.1 (4)
Caretta caretta	Loggerhead turtle	4.46 (1)	2.3 (1)
Balaenoptera acutorostrata	minke whale	3.39 ± 0.90 (6)	2.4 ± 0.1 (3)
Balaenoptera borealis	sei whale	6.13 ± 1.46 (3)	2.5 ± 0.2 (3)
Balaenoptera physalus	fin whale	2.50 ± 0.15 (3)	2.5 ± 0.1 (3)
Grampus griseus	Risso's dolphin	4.28 ± 1.23 (3)	-
Kogia breviceps	Pygmy sperm whale	2.11 ± 0.44 (6)	-
Kogia sima	Dwarf sperm whale	3.69 ± 0.66 (6)	-
Mesoplodon bidens	Sowerby's beaked whale	2.83 ± 0.71 (3)	-
Mesoplodon densirostris	Blainville's beaked whale	2.57 ± 0.57 (3)	-
Mesoplodon europaeus	Gervais' beaked whale	2.54 ± 1.08 (6)	-
Hippopotamus amphibius	hippopotamus	2.02 (1)	2.44 (1)
Bos taurus	cow	0.81 (1)	2.1 (1)
Capra aegagrus hircus	goat	5.4 (1)	2.5 (1)
Ovis aries	sheep	5.38 (1)	2.3 (1)
Sus scrofa	pig	1.43 ± 0.23 (2)	2.3 ± 0.01 (2)
Overall		3.56 ± 1.67 (54)	2.4 ± 0.2 (24)

Overall the turtles and seabirds had the highest microvascularity, and the toothed whales had the lowest (Figure 7). There were significant differences across animal groups in the amount of microvessels present (ANOVA; $P < 0.001$), even if the acoustic fats were excluded (ANOVA; $P<0.001$) – it appears that the acoustic fats also differ from normal storage depots in microvascularity in addition to nitrogen solubility. In contrast, degree of branching did not vary across animal groups (ANOVA; $P = 0.618$) with most animals having 2.3 – 2.4 branches per microvessel.

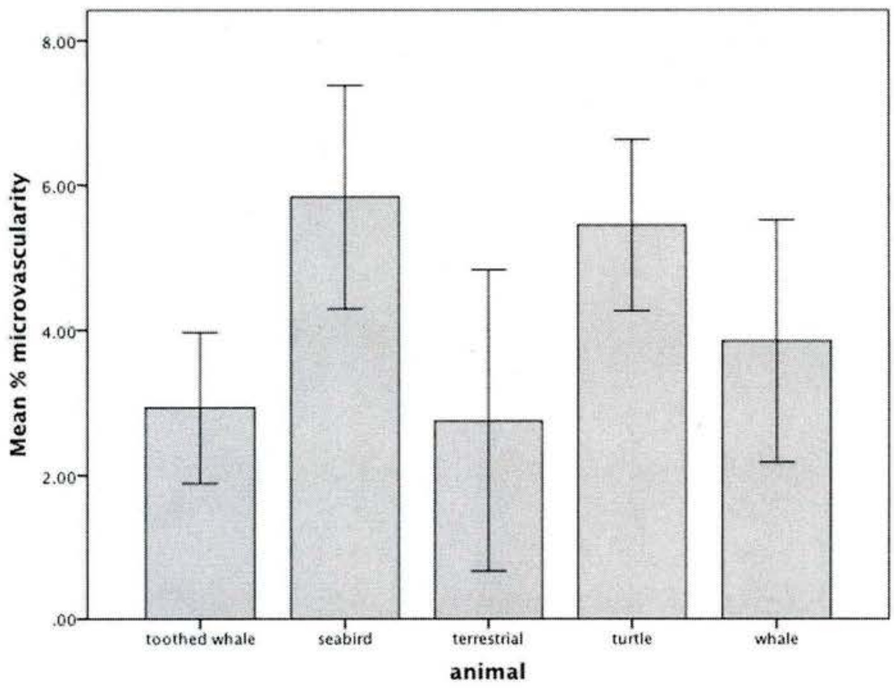

Figure 7. Mean % microvascularity (± standard deviation) for storage depots (adipose, blubber) in animals of different taxonomic groups.

In the non-acoustic tissues, there was a trend towards a positive relationship between % microvascularity and degree of branching (Figure 8) but this was not significant (P = 0.057).

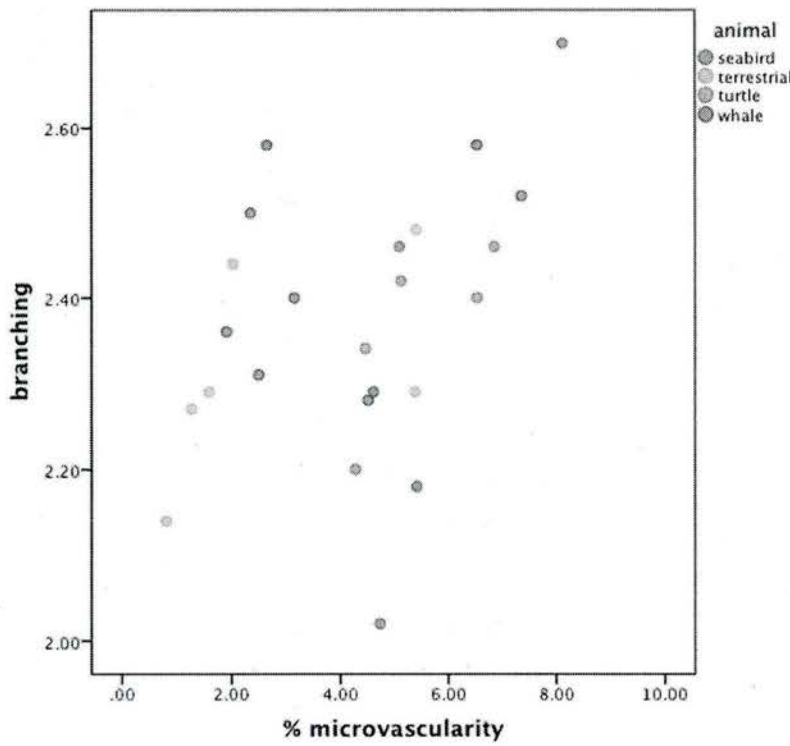

Figure 8. Relationship between number of microvessels and degree of branching in storage depots (adipose).

Table 4. Microvascularity (% of area of tissue occupied by microvessels) and degree of branching in blubber and acoustic fats of toothed whales (Odontocetes). Values are means for the species ± standard deviation. Sample sizes are given in parentheses.

Species	% Microvascularity			Branching		
	blubber	EMFB	IMFB	blubber	EMFB	IMFB
Bottlenose dolphin (*Tursiops truncatus*)	-	1.12 (1)	1.59 (1)	-	2.1 (1)	2.3 (1)
Atlantic spotted dolphin	-	1.52 ± 0.25 (3)	1.17 ± 0.25 (3)	-	2.2 ± 0.02 (3)	2.1 ± 0.04 (3)
Short-finned pilot whale	-	1.00 ± 0.15 (3)	1.07 ± 0.24 (3)	-	2.2 ± 0.1 (3)	2.2 ± 0.04 (3)
Risso's dolphin (*Grampus griseus*)	4.28 ± 1.23 (3)	1.58 (1)	1.68 (1)	-	2.2 (1)	2.4 (1)
Pygmy sperm whale	2.11 ± 0.44 (6)	1.17 ± 0.19 (3)	1.01 ± 0.01 (2)	-	2.1 ± 0.03 (3)	2.1 ± 0.1 (3)
Dwarf sperm whale (*K. sima*)	3.69 ± 0.66 (6)	1.22 ± 0.35 (2)	-	-	2.2 ± 0.01 (2)	-
Sowerby's beaked whale (*M. bidens*)	2.83 ± 0.71 (3)	1.14 (1)	0.51 (1)	-	2.1 (1)	2.1 (1)
Blainville's beaked whale	2.24 ± 0.14 (2)	0.75 (1)	1.15 (1)	-	2.1 (1)	2.5 (1)
Gervais' beaked whale	2.54 ± 1.08 (6)	0.72 ± 0.22 (2)	0.70 ± 0.08 (2)	-	2.1 ± 0.1 (2)	2.1 ± 0.04 (2)
True's beaked whale (*M. mirus*)	-	0.89 (1)	0.57 (1)	-	2.1 (1)	2.0 (1)
Cuvier's beaked whale (*Ziphius cavirostris*)	-	0.57 (1)	0.75 (1)	-	2.1 (1)	2.1 (1)

For toothed whales, % microvasculature varied by tissue type, with blubber having significantly more (ANOVA; $P < 0.001$) vessels (mean 2.93 ± 0.14) than either the Intra- (1.03 ± 0.18) or Extra-mandibular (1.11 ± 0.17) fat bodies (which were not different from each other). Overall the acoustic fats had the lowest % microvascularity of all fat depots (compare the overall adipose mean of 3.54 [Table 3] with the acoustic fat means of ~ 0.76 [see below]). We did not have any data for branching in blubber, but there was no difference between the fat bodies ($P = 0.371$). However within the acoustic fats, there was a significant positive relationship between % vascularity and degree of branching

(Figure 9; $P = 0.001$; $R^2 = 0.273$), suggesting that as the density of small vessels increases, branching increases – although whether this is a functionally significant pattern is unknown, given the small range over which this change in branching occurs (2.1 – 2.4; Figure 9).

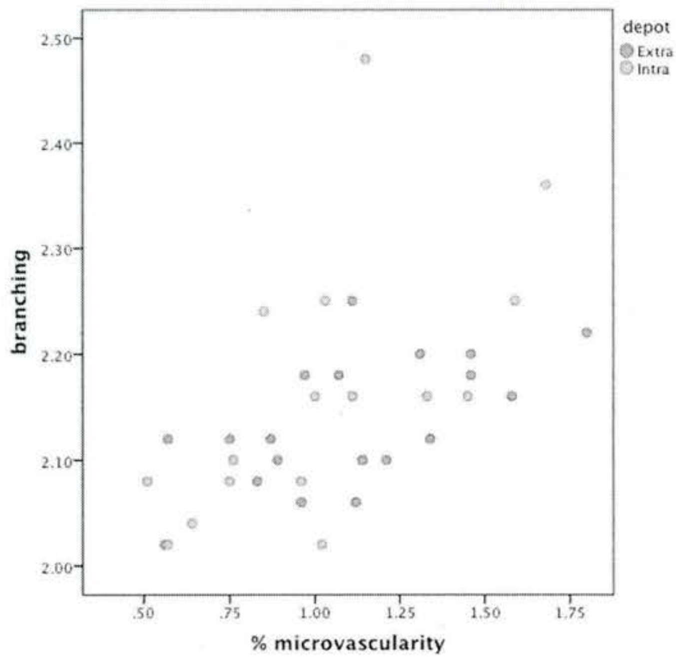

Figure 9. Relationship between amount of microvasculature and degree of branching in toothed whale acoustic tissues.

The higher degree of microvascularity in the blubber, and the similar (but lower) values in the two types of acoustic fat bodies, can be easily seen in Figure 10. What is also notable here is that there is a slight decrease in % microvascularity in the acoustic fats, moving from the left side to the right side of the figure. The species are arranged in the same order as in Table 4, which is phylogenetically (dolphins, small sperm whales, and beaked whales) but also reflects the general diving patterns of these animals. If the toothed whales are divided into shallow divers (*Tursiops, Stenella*), intermediate divers (*Globicephala, Grampus, Kogia*) and deep divers (*Mesoplodon and Ziphius*), the acoustic fats of the deep divers (0.76 ± 0.07) have significantly lower % microvascularity (ANOVA; $P < 0.001$) than both the intermediate (1.16 ± 0.07) and shallow divers (1.35 ± 0.09).

Previous data obtained by our lab on microvascularity of blubber (McClelland et al. 2012, data from our first ONR grant) showed that microvascular density varies across species, and blubber depth. The blubber of *Tursiops* (bottlenose dolphins) had % microvascularity values between 3.3 – 9.3 (greater values in the deeper parts of the blubber), *Kogia* (pygmy sperm whale) had 3.3 – 4.5 (again, more in the deeper layers), and beaked whales (*Mesoplodon* and *Ziphius*) had between 1.5 – 2.8 (McClelland et al. 2012). It would appear that deeper diving toothed whales tend to have lower microvascular density values in both blubber and acoustic fat bodies. One factor that is poorly understood in these animals is whether, and how, the lipids of the blubber are mobilized in times of energy need. It has been assumed that the acoustic fats are "metabolically inert" (so termed because they are not depleted in fasting or starvation, e.g. Koopman et al. 2003) but we have little information on seasonal/fasting/reproductive changes in blubber adipocytes or total lipid content in toothed whales beyond harbour porpoises (*Phocoena phocoena*) and *Tursiops*, both of which have been shown to deplete blubber lipids during periods of negative energy balance (Koopman et al. 2002; Struntz et al. 2004). Beaked whales and sperm whales may not make use of their blubber lipids (possibly because wax esters are difficult for mammals to metabolize; see Swaim et al. 2009 and Koopman 2007). In our lab we are currently quantifying basic adipocyte size and shape in the blubber of these latter animals so that changes in emaciated animals can be evaluated against a "healthy" baseline.

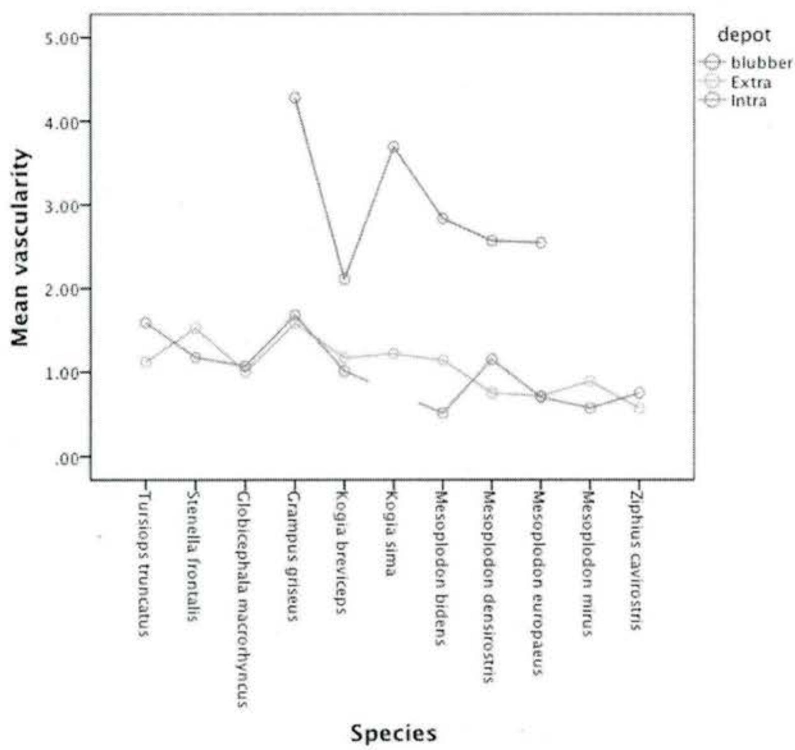

Figure 10. % microvascularity of blubber and acoustic fats of toothed whales, coded by species.

Finally, Figure 11 shows some good examples of the diversity in the degree of small blood vessels in the species examined in this study. Note the 8-fold difference in the amount of microvascularity between the leatherback sea turtle (B) and the acoustic fats of the Gervais' beaked whale (C and D).

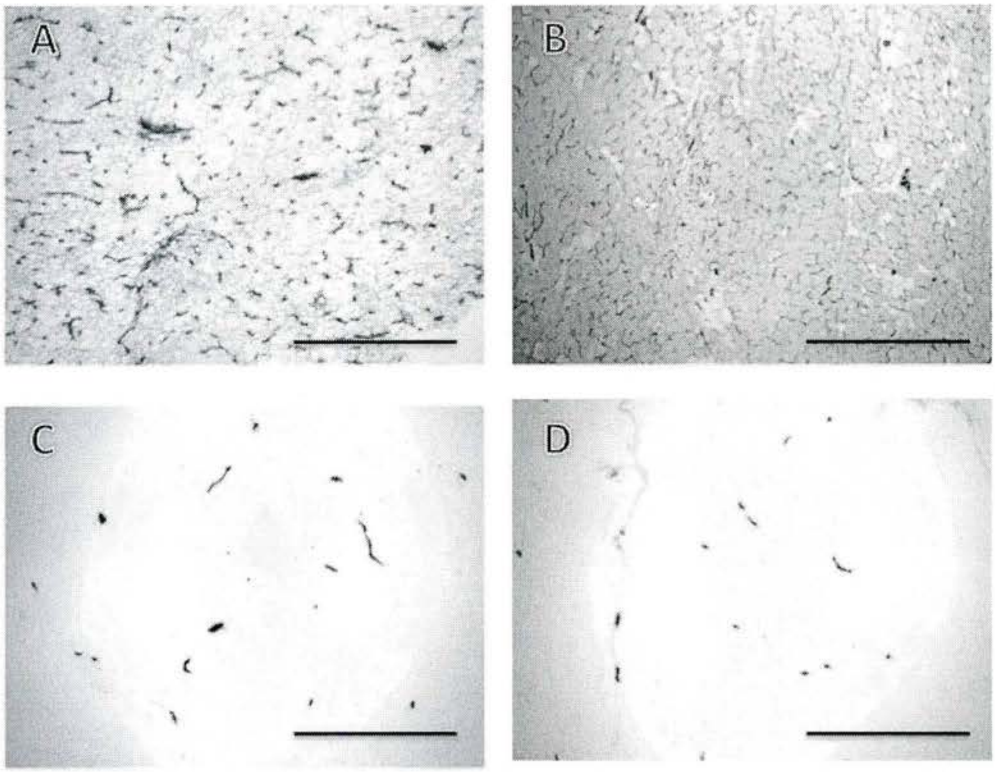

Figure 11. Microvascularity in representative adipose tissues. A – leatherback sea turtle adipose, B – goat back fat, C – Gervais' beaked whale extramandibular fat body, D – Gervais' beaked whale intramandibular fat body. Scale bar 1 mm.

Relationship between nitrogen solubility and microvasculature

Across all samples, there was a trend towards decreasing nitrogen solubility in tissues with greater microvascularity (Figure 12). This relationship was significant ($P < 0.001$; $R^2 = 0.508$) such that the density of microvessels explained half the variation in nitrogen solubility. It should be noted that almost all the samples for which we had both blood vessel and solubility data in Figure 12 are in fact diving tetrapods. Therefore across air-breathing divers, solubility and the density of small blood vessels may be mutually constrained. The functional significance of this is not known. Does high nitrogen solubility mean a greater risk of N_2 loading and thus the amount of potential exchange surface with the blood needs to be reduced? It is also possible that these features are unrelated. The density of microvessels can reflect many selective pressures: delivery and removal of gas (O_2 and CO_2), nutrient delivery and removal, or thermoregulatory needs (although one might suspect that larger blood vessels, such as arteries and veins, would serve this role). As mentioned above, there may be a range of adipose function across these species, and some animals may not need to rapidly mobilize lipid from adipose. We know that seabirds and turtles do so seasonally and in association with reproductive events (e.g. Groscolas 1990), and thus it makes sense that they would have greater amounts of small vessels for this to occur. We also know that large baleen whales (fin and sei whales) mobilize blubber lipids during reproduction, especially for lactation (Lockyer 1986). As mentioned above, whether the deep diving toothed whales do this is unknown and further investigation into patterns of lipid use are needed to fully interpret these data.

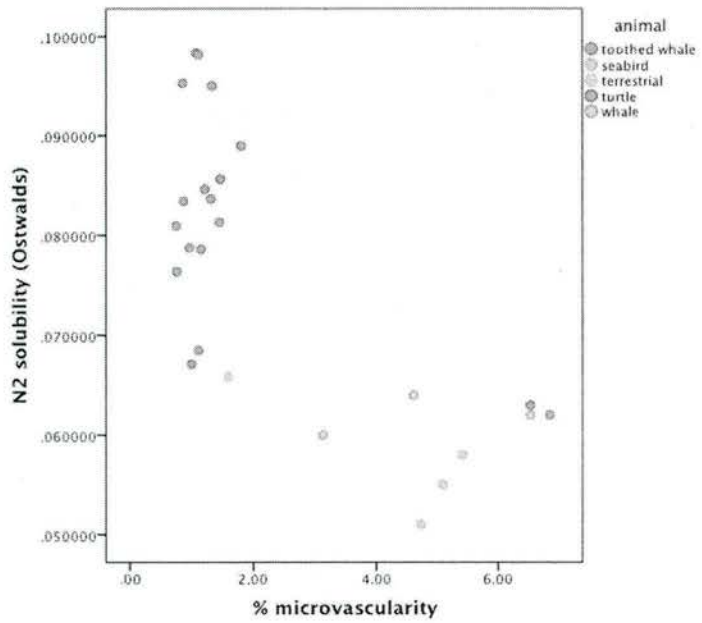

Figure 12. Nitrogen solubility decreases as the density of microvessels increases in adipose depots of tetrapods.

One possibility is that high nitrogen solubility, whether as an evolved feature or a consequence of lipid composition for another function (acoustics, insulation, buoyancy) has led to a reduction in the density of small vessels as a tradeoff to reduce nitrogen loading during deeper dives in the acoustic fats of toothed whales, and the wax-rich blubber of beaked and sperm whales. This idea is purely speculative and requires information on control of blood flow, temperature and phase of these tissues during all parts of a dive (including exposure to cooler temperatures) as well as nitrogen partitioning throughout the body. However as lipid has ~ 5 times the nitrogen solubility of water/blood (and up 7 times if some of the acoustic fats are considered), if give the opportunity (at an exchange surface), nitrogen gas will move from blood to fat, making it a reasonable hypothesis that high N_2 solubility might constrain the density of small exchange vessels where gases can interact with the bloodstream.

Most important conclusions

- There is considerable variation across species/animal groups in nitrogen solubility, lipid composition, and microvascular components among diving tetrapods and terrestrial animals.
- While for most species (those lacking wax esters and short/branched chain fatty acids and alcohols) it may be appropriate to use a "standard" value for nitrogen solubility around our overall mean of 0.064, this does not apply to all animal groups (for example, consider the low values for the seabirds, ~ 0.055). Therefore it is important to obtain at least representative nitrogen solubility values for taxonomic groups not yet examined.
- Nitrogen solubility values for the human and pig samples were very similar, making it tempting to use an Ostwald number of ~ 0.064 – 0.065 for any diving physiology models including adipose as a compartment, be they for humans or for mammalian models commonly used in such studies. However we advise caution here, especially given the small samples size and the variability in solubility values among the non toothed whale marine species, which we would expect to have fairly similar lipid compositions.
- Toothed whale acoustic fats have higher nitrogen solubility and less microvasculature than other depots. The link between these data, if any, is unknown at this time. Whether these chemical and anatomical features represent adaptations to or complexities for diving physiology is also unknown, but it may be that these tissues, under certain pressure circumstances, may (we stress "*may*" here) experience higher nitrogen loads while diving. If so, this could mean that some species have greater probabilities of experiencing DCS, particularly if their normal diving patterns and thus pressure regimes are disturbed.
- Temperature likely affects nitrogen solubility of biological oils, although this element of the study needs additional work.

Future directions

We would like to continue to develop many of the ideas in this project, including:

- The effects of biologically relevant temperatures on the nitrogen solubilities of these oils, focusing particularly on what happens during a dive.
- Expanding the project to include nervous tissues, as these are lipid-rich and extremely sensitive to changes in pressure, with bubble formation here being a major health concern
- Linking the degree of vasculature with the amount of actual blood flow through adipose during a dive. We have established that there is variation in the amount of surface available for exchange between blood and adipose, but we do not know how this is utilized under different conditions.

VI. Work Plan

Lipid Extraction and Analysis – Lipids were extracted from adipose using a modified Folch procedure (Folch et al., 1957; Koopman et al., 1996). Samples were trimmed to remove any desiccated tissues and all epidermis and muscle/connective tissue. The bulk of the extracted oil was used for the nitrogen solubility measurements, and a small aliquot

(~ 0.5 mL) was reserved for analyses of lipid profiles. Lipid classes were separated and quantified via thin-layer chromatography-flame ionization detection (TLC-FID). Samples will be spotted on chromarods (Chromarod-SIII, Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) and developed in 94/6/1 hexane/ethyl acetate/formic acid. Classes were quantified by TLC-FID using an Iatroscan MK-6 (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan). Identification was confirmed through use of lipid class standards (Nu Chek Prep, Elysian, MN). Peaks were integrated with Peaksimple software (Peaksimple 3.29, SRI Instruments, Torrance, CA). For gas chromatography (GC) analysis, fatty acid butyl esters (FABE) were prepared from total lipid extracts (see Koopman et al. 1996). Butyl esters were used as opposed to methyl esters (more commonly used) because short-chained fatty acids are volatile (Koopman, 2007). Fatty acids/alcohols were analyzed by GC using a Varian capillary GC (3800) with a flame ionization detector (FID) in a 30m x 0.25mm column coated with nitroterephthalic acid modified with polyethylene glycol (Zebron, ZB-FFAP column) with He as the carrier gas. The following temperature program was used: 65°C for 2 min, hold at 165°C for 0.40 min after ramping at 20°C/min, hold at 215°C for 6.6 min after ramping at 2°C/min, and hold at 250°C for 5 min after ramping at 5°C/min. Up to 80 different fatty acids/alcohols were identified following Koopman (2007) and Koopman and Zahorodny (2008). Peak identification was based on comparisons of retention time to standards (Nu Chek Prep, Elysian, MN) and known samples. Peaks were integrated using appropriate response factors with Galaxie Chromatography Data System (Version 1.8.501.1, Varian Inc., Palo Alto, CA), and peak identification was manually confirmed for each run.

Nitrogen Solubility – Through a previous grant from ONR (N00014-06-0308; Koopman and Westgate 2012), our lab spent several years developing a new method (based on information from Snedden et al. 1996) for measuring nitrogen solubility in oils by incubating oils and pure nitrogen gas in headspace vials inside a sealed environment (glove box with argon) and quantifying N_2 liberated from the oils post-incubation via gas chromatography (GC). Solubility experiments for this grant took place in an Argon environment in a sealed glove box to minimize N_2 contamination. Prior to and during each solubility run, background levels of nitrogen were monitored by injecting samples of glove box gas phase onto the GC column as described below for experimental samples. Approximately 3-6 mL of test oil was placed into a 10 cc airtight Hamilton syringe. N_2 was bubbled through the oil for 30 minutes through a port in the bottom of the syringe. The oil is then removed and placed into two (one 10 and one 20 mL) preweighed headspace vials for incubation. Early results from our previous work indicated that the system was highly sensitive to slight changes (timing, vial handling, etc.) or minute contamination (in vials, needles and the glove box) and to ensure accurate results, each run is carried out in duplicate with both 10 and 20 ml headspace vials to catch any false results (i.e. the two vials had to produce the same solubility values for the run to be considered successful; runs in which the two were not equal were discarded). Precise oil volume were later calculated by weighing the oil and using its density. Density will be determined separately for each oil by weighing the amount of oil in a 5 ml volumetric flask. The syringe and headspace incubation vials were kept at constant temperature during the three hour incubation period by surrounding vials with coils containing water circulating from a Fisher waterbath; with this system temperature varied

<0.1 °C. Following incubation, the gas phase from each vial was sampled using a 25 μl Hamilton syringe through a port in the glove box that permits direct sampling through the seal on the headspace vial without the need to disrupt the glove box environment. The headspace gas sample was hand-injected onto a 25 m Molsieve 5A column running isothermal at 50 °C with He as the carrier gas on a Varian GC (3800). Peaks were quantified using a thermal conductivity detector (TCD) and identified and integrated using Galaxie GC software (version 1.8, Varian, Inc.). Peak areas were then converted to N_2 volume using the standard curve generated from known volumes of N_2. Nitrogen solubility values were calculated as Ostwald coefficients (mL gas dissolved/mL oil) using mass balance.

For our standard set-up we ran the bubbling and incubation at 37 °C. For the temperature-response experiments, we had planned to run these at a range of biologically relevant temperatures (~4 to 37 °C); however we encountered some difficulties at temperatures lower than ~30 °C that caused us to have some concerns about the results in this section of the study.

In Year One we proposed to build a second syringe/incubation set-up that would permit two samples to be run at the same time to greatly enhance productivity. This second system was completed and the net result was that we ran each sample in quadruplicate each day. This improved our sensitivity and lowered variation in each sample's overall nitrogen solubility.

Measures of Microvascularity – Frozen, unfixed tissue from all animals was placed in a Leica Cryocut 1800 (Leica Microsystems Inc., Bannockburn, IL), covered with Optimal Cutting Temperature Compound (OCT) (Ted Pella, Inc., Redding, CA), and allowed to freeze to approximately -27°C (temperature determined through preliminary trials with blubber samples). Each tissue subsample was sectioned until five noncontiguous 30micrometer thick sections were obtained. The exception to this process was the human adipose tissue, which was fixed in formalin prior to sectioning to minimize exposure to disease agents, and was embedded in paraffin and sectioned with a rotary microtome. Controls with fixed and frozen tissue from selected animal species were used to verify that fixing did not cause any artifacts in processing. Sections were rinsed in Sorensen's phosphate-buffered saline (PBS) for 15 minutes (Presnell and Schreibman, 1997), and then incubated in a nitro blue tetrazolium chloride plus 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution made out of NBT/BCIP ready-to-use tablets dissolved in 10ml of distilled water (Roche Diagnostics, Mannheim, Germany). NBT/BCIP stains for alkaline phosphatases, a family of enzymes, which are located in the microarterioles, microvenules, and capillaries (Foley et al., 1954; McComb and Bowers, 1979; Hansen-Smith et al., 1992) and has been used to view blood vessels in people, rats, rabbits, pigs and other mammals (Foley et al., 1954; Wachstein and Meisel, 1959; Cros et al., 1980; Hausman and Richardson, 1983; Werner et al., 1987). After incubation with NBT/BCIP, sections were rinsed in PBS for seven minutes and placed under coverslips with trisglycerol (Presnell and Schreibman, 1997). Stained sections were viewed with an Olympus BX60 (Olympus America Inc., Center Valley, PA) and digital pictures were taken of each section using a Diagnostic Instruments SPOT RT digital camera (Diagnostics Instruments, Sterling Heights, MI). All images were analyzed using Image ProPlus software (Media Cybernetics, Inc., Bethesda, MD). The following variables were measured: percent vascularity, vessel size, and vessel branching. These methods were developed and successfully used for our preliminary analysis of marine mammal blubber and pig adipose tissue (McClelland et al. 2012).

Analysis – Our goals were to link 1) nitrogen solubility with lipid composition; 2) evaluate the degree of microvasculature across several groups of diving tetrapods; 3) evaluate the relationship between temperature and nitrogen solubility for biological oils.

Graduate Student Training/Mentorship – This project supported a PhD student (Gabler), who carried out the majority of the chemistry, histology and solubility work. Koopman was the primary mentor of this student, providing training in scientific inquiry, advising on classes, and mentoring the student through the steps of graduate school and writing papers/designing presentations for conferences. Westgate provided guidance to the student in the form of training in use and maintenance of all lab instrumentation, especially the solubility apparatus. Westgate also served as mentor for the student in terms of project design, and generation of reports, manuscripts and presentations. Gabler has completed three years of her five year program, and will publish at least three manuscripts from the work related to this project as part of her dissertation. This project also supported the work of a M.Sc. student (Lonati), who worked on the nitrogen solubility and lipid composition of the acoustic fats of toothed whales. Three other students (1 M.Sc., 2 undergraduates) worked on other aspects of the lipid composition or physical properties of the same samples, providing additional training/mentorship opportunities.

VII. Major Problems/Issues

We encountered two problems over the course of this study. The first problem was access to human adipose tissues. This was a nagging issue for the first three years of the project. At the onset of this project (at the proposal stage), a surgeon from UNC-Chapel Hill agreed to assist and provide us with tissue, and all necessary paperwork was in place. Approximately a year after the project started, that surgeon left UNC-CH for another institution, taking with him the institutional memory of agreeing to collaborate with us. We attempted to find a way to obtain tissue from another surgeon and were thwarted on many fronts and layers of bureaucracy, including spending 6 months trying to find a way into the UNC-CH ethics/privacy website so I could be approved, only to discover from a different UNC-CH administrative office that this was not needed. We finally (in April 2015) found another surgeon at UNC-CH willing to assist, and the paperwork was again submitted for approval for us to use waste adipose tissue (in July 2015). As of fall 2015 we still had not been able to access any adipose samples and decided to arrange an in-person meeting with the surgeon, her post-doc and the tissue bank manager on the UNC-CH medical school campus in Chapel Hill. This was a productive meeting and we finally obtained access to our first sample in spring 2016.

The second issue was related to our goal of determining how nitrogen solubility in biological oils changed with temperature, under the premise that diving animals will experience variation in water temperature with depth, and that even homeothermic endotherms (birds and mammals) would be subject to differing tissue temperatures, particularly those at the periphery (such tissues are often adipose). We conducted several trials with olive oil (see expanded accomplishments [V.] above) and got a clear pattern of decreasing solubility in cooler temperatures, but when we moved to animal oils we were not entirely confident that the changes we were seeing with temperature were not related to an increase in viscosity of the oils, which could have led to under-saturation with nitrogen at the bubbling stage, yielding a lower Ostwald value. We therefore have some preliminary data, but did not collect enough data points to have successfully accomplished this objective. Additional trials with different bubbling regimes would be required to better establish any trends in nitrogen solubility under cooler conditions.

VIII. Technology Transfer
N/A

IX. Foreign Collaborations and Supported Foreign Nationals
Dr. Heather Koopman, UNCW (PI for project, took partial month of salary at tend of project to compile data). Canadian citizen/U.S. Permanent Resident. Naturalized U.S. citizen September 2016.
Dr. Andrew Westgate, UNCW (on salary; conceived and assembled nitrogen apparatus, ran all samples). Canadian citizen/U.S. Permanent Resident. Naturalized U.S. citizen February 2016.

X. Productivity (students in bold)
A. Refereed Journal Articles

Lonati, G.L., Westgate, A.J., Pabst, D.A. and Koopman, H.N. 2015. Nitrogen solubility in odontocete blubber and mandibular fats in relation to lipid composition. Journal of Experimental Biology 218: 2620-2630.

Gabler, M.K., Gay, D.M., Westgate, A.J. and Koopman, H.N. *In preparation*. Microvascular characteristics of the acoustic fats of odontocetes: a comparison between deep- and shallow-diving species. Journal of Morphology.

Gabler, M.K., Gay, D.M., Westgate, A.J. and Koopman, H.N. *In preparation*. Microvascularity of adipose tissue in diving tetrapods. Journal of Morphology.

Gabler, M.K., Gay, D.M., Westgate, A.J. and Koopman, H.N. *In preparation*. Nitrogen solubility in the adipose of diving tetrapods.

B. Non-refereed Significant Publications
N/A

C. Books or Chapters
N/A

D. Technical Reports
N/A

E. Workshops and Conferences

Yanes, A. and Koopman, H. (2016). Composition and Distribution of Intact Waxes in the Acoustic Fats of Toothed Whales. Oral presentation delivered at the Meeting of the Southeast and Mid-Atlantic Marine Mammal Symposium, Savannah, GA April 3, 2016.

Gabler, M.K., Gay, D.M., Westgate, A.J., and Koopman, H.N. (2015). Microvascular characteristics of the acoustic fats of deep and shallow-diving odontocetes. Oral presentation delivered at the Biennial Conference on the Biology of Marine Mammals, San Francisco, CA, December 13-18, 2015.

Koopman, H.N., Westgate, A.J., **Ernst, T.R., Yanes-Belardi, A., Lonati, G.L.** and **Gabler, M.K.** (2015). Acoustic fat bodies of beaked whales: Unexpected variation in physical and biochemical properties within the Ziphiids. Oral presentation delivered at the Biennial Conference on the Biology of Marine Mammals, San Francisco, CA, December 13-18, 2015.

Yanes, A. and Koopman, H. (2015). Composition and distribution of intact waxes in the acoustic fats of toothed whales. Poster at the Society for Marine Mammalogy 21st Biennial Meeting, San Francisco, Dec 13-18.

Gabler, M.K., Gay, D.M. Westgate, A.J. and Koopman, H.N. (2015). A comparative study of the microvascularity of adipose in a variety of diving tetrapods and terrestrial mammals. Oral presentation delivered at the Annual Meeting for the Society for Integrative and Comparative Biology, West Palm Beach, FL, January 3-7, 2015.

Pelletier, R. C., Heather N. Koopman. (2015). Heterogeneity in the acoustic fat deposits in the narwhal, *Monodon monoceros*. SEAMAMMS (Southeast and Mid-Atlantic Marine Mammal Symposium) meeting, March 27-29,2015. Virginia Beach, VA.

Ernst, T., A. Westgate, **G. Lonati** and H. Koopman. (2015). Melting points of acoustic fats from diving marine mammals. SEAMAMMS (Southeast and Mid-Atlantic Marine Mammal Symposium) meeting, March 27-29, Virginia Beach, VA.

Gabler, M.K., Gay, M. and Koopman, H.N. (2014). Microvascularization of the extra and intra mandibular acoustic fat bodies in odontocetes. Poster presentation delivered at the Meeting of the Southeast and Mid-Atlantic Marine Mammal Symposium, Wilmington, NC, March 28-30, 2014.

Lonati, G. L., Westgate, A. J., and Koopman, H. N. (2014). Differences in lipid composition may influence nitrogen solubility and risk of barotrauma in odontocete intramandibular and extramandibular fats. SEAMAMMS (Southeast and Mid-Atlantic Marine Mammal Symposium) meeting, March 28-30, Wilmington, NC.

Lonati, G. L., Westgate, A. J., and Koopman, H. N. (2014). Nitrogen solubility related to lipid composition in toothed whale fats. Society for Integrative and Comparative Biology meeting, Austin TX Jan 3-7. *This presentation was awarded the* ***DCPB Jeffrey B. Graham student presentation award.***

Lonati, G. L., Westgate, A. J., and Koopman, H. N. (2013). Variation in lipid composition of odontocete acoustic fats may affect gas loading during dives. Society for Marine Mammalogy 20th Biennial Meeting, Dunedin New Zealand, December 9-13.

Koopman, H. N. (2013). The evolution of acoustic lipids in Odontocetes. Society for Marine Mammalogy 20th Biennial Meeting, Dunedin New Zealand, December 9-13.

F. Patents
N/A

G. Awards/Honours
Our **Lonati** et al. paper was selected by the Editor as one of the papers in the issue to highlight in their Inside JEB section (http://jeb.biologists.org/content/218/16/2491), and was also picked up by National Geographic Online (http://news.nationalgeographic.com/2015/08/150819-whales-dolphins-bends-decompression-sickness/).
In addition, the talk Gina **Lonati** gave at the 2014 Society for Integrative and Comparative Biology meeting in Austin, TX was selected for the DCPB (Division of Comparative Physiology and Biochemistry) Jeffrey B. Graham award for best student oral presentation.

XI. Award Participants

Dr. Heather Koopman, UNCW (PI for project, took partial month of salary at tend of project to compile data).
Dr. Andrew Westgate, UNCW (on salary; conceived and assembled nitrogen apparatus, ran all samples)
Molly Gabler, UNCW (PhD student, on stipend; project involves nitrogen solubility in diving tetrapods and associated microvasculature)
Gina Lonati, UNCW (MSc Student, completed thesis on nitrogen solubility in acoustic fats of toothed whales)
Ana Yanes, UNCW (MSc Student, completed thesis on lipid composition of acoustic fats of toothed whales)

References for this Report

Blomberg, J., and L.-E. Lindholm, L.-E. 1976. Variations in lipid composition and sound velocity in melon from the North Atlantic pilot whale *Globicephala melaena melaena*. Lipids 11: 153–156.

Carturan, D., Boussuges, A., Vanuxem, P., Bar-hen, A., Burnet, H., and Gardette, B. 2002. Ascent rate, age, maximal oxygen uptake, adiposity, and circulating venous bubbles after diving. Journal of Applied Physiology 93: 1349-1356.

Crandall, D.L., Hausman, G.J., and J.G. Kral. 1997. A Review of the microcirculation of adiposetissue: anatomic, metabolic, and angiogenic perspectives. Microcirculation 4: 211-232.

Cros, D., Pearson, C., and M.A. Verity. 1980. Polymyositis-Dermatomyositis diagnostic and prognostic significance of muscle alkaline phosphatase. American Journal of Pathology 101: 159-176.

Dembert ML, Jekel JF, Mooney LW. 1984. Health risk factors for the development of decompression sickness among U.S. Navy divers. Undersea Biomed Res. 11(4):395-406.

Douglas, J. D. 1985. Medical problems of sport diving. British Medical Journal (Clinical Research and Education) 291:1224-1226.

Farhi, L. E., Edwards, A. W. T., and Homma, T. 1963. Determination of dissolved N2 in blood by gas chromatography and (a-A)N_2 difference. Journal of Applied Physiology 18: 97-106.

Folch, J., Lees, M., and G.H. Sloan-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226: 497-509.

Foley, R.C., Reece, R.P., and J.H. Leathem. 1954. Histochemical observations of the bovine uterus, placenta, and corpus luteum during early pregnancy. Journal of Animal Science 13: 131-137.

Gersh, I.G., and M.A. Still. 1945. Blood vessels in fat tissue relation to problems of gas exchange. Journal of Experimental Medicine 81: 219-232.

Gerth, W.A. 1985. Applicability of Henry's law to hydrogen, helium, and nitrogen solubilites in water and olive oil at 37°C and pressures up to 300 atmospheres. *Arch Biochem Biophys* 241:187-199.

Groscolas, R. 1990. Metabolic adaptations to fasting in emperor and king penguins. In *Penguin Biology*, ed. L. S. Davis and J. T. Darby, 269-296. San Diego, CA: Academic Press.

Hansen-Smith, F.M., Blackwell, L. H., and G. R. Joswiak. 1992. Expression of muscle capillaryalkaline phosphatase is affected by hypoxia. Journal of Applied Physiology 73: 776-780.

Hausman, G.J., and R.L. Richardson. 1983. Cellular and vascular development in immature ratadipose tissue. Journal of Lipid Research 24: 522-532

Hausman, G.J. and J.T. Wright. 1996. Cytochemical studies of adipose tissue associated bloodvessels in untreated and thyroxine treated hypophysectomized pig fetuses. Journal of Animal Science 74: 354-362.

Herd, J.A., Goodman, H.M., and S.A. Grose. 1968. Blood flow rates through adipose tissue of unanesthetized rats. American Journal of Physiology 214: 263-268.

Ikels, K. G. 1964. Determination of the solubility of nitrogen in water and extracted human fat. Technical Report, USAF School of Aerospace Medicine. SAM-TR 94: 1-9.

Koopman, H.N. 2007. Phylogenetic, ecological, and ontogenetic factors influencing the biochemical structure of the blubber of odontocetes. Marine Biology 151: 277-291.

Koopman, H. N., Budge, S. M., Ketten, D. R., Iverson, S. J. 2006. The topographical distribution of lipids inside the mandibular fat bodies of odontocetes: remarkable complexity and consistency. IEEE Journal of Oceanic Engineering 31:95–106

Koopman, H.N., Iverson, S.J., and D.E. Gaskin. 1996. Stratification and age-related differences in blubber fatty acids of the male harbour porpoise (*Phocoena phocoena*). Journal of Comparative Physiology B 165: 628-639.

Koopman, H. N., Iverson, S. J., and Read, A. J. 2003. High concentrations of isovaleric acid in the fats of odontocetes: Variation and patterns of accumulation in blubber vs. stability in the melon. Journal of Comparative Physiology B 173: 247–261.

Koopman, H. N., Pabst, D. A., McLellan, W. A., Dillaman, R. M., and Read, A. J. 2002. Changes in blubber distribution and morphology associated with starvation in the harbor porpoise (*Phocoena phocoena*): evidence for regional differences in blubber structure and function. Physiological Zoology 75: 498-512.

Koopman, H. N., and Westgate, A. J. 2012 Solubility of nitrogen in marine mammal blubber depends on its lipid composition. Journal of Experimental Biology 215: 3856-3863.

Koopman, H. N. and Zahorodny, Z. P. 2008. Life history constrains biochemical development in the highly specialized Odontocete echolocation system. Proceedings of the Royal Society of London, B 275:2327-2334.

Lam TH, Yau KP. 1989. Analysis of some individual risk factors for decompression sickness in Hong Kong. Undersea Biomed Res. 16:283-92.

Langø, T., Mørland, T., and Brubakk, A. O. 1996. Diffusion coefficients and solubility coefficients for gases in biological fluids and tissues: a review. Undersea and Hyperbaric Medicine 23: 247-272.

Litchfield, C., Greenberg, A. J., Caldwell, D. K., Caldwell, M. C., Sipos, J. C., and Ackman, R.G. 1975. Comparative lipid patterns in acoustical and nonacoustical fatty tissues of dolphins, porpoises and toothed whales. Comparative Biochemistry and Physiology B 50: 591-597.

Lockyer, C. 1986. Body fat condition in Northeast Atlantic fin whales, *Balaenoptera physalus*, and its relationship with reproduction and food resource. Canadian Journal of Fisheries and Aquatic Sciences 43: 142-147.

Lonati, G.L., Westgate, A.J., Pabst, D.A. and Koopman, H.N. 2015. Nitrogen solubility in odontocete blubber and mandibular fats in relation to lipid composition. Journal of Experimental Biology 218: 2620-2630.

McClelland, S. J., Gay, M., Pabst, D. A., Dillaman, R., Westgate, A. J., and Koopman, H. N. 2012. Microvascular patterns in the blubber of deep and shallow diving Odontocetes. Journal of Morphology 273:932-942.

McComb, R.B., Bowers, G.J.N., and S. Posen. 1979. Alkaline phosphatase. Plenum Press, New York, NY.

Morris, R. J. 1986. The acoustic faculty of dolphins. Pages 369–399 in Research on Dolphins. M. M. Bryden and R. Harrison, Eds. Oxford, U.K.: Clarendon Press.

Norris, K. S. 1968. The evolution of acoustic mechanisms in odontocete cetaceans. Pages 297-324 in Evolution and Environment, E. T. Drake, Ed. New Haven, CT: Yale Univ. Press.

Presnell, J.K. and M.P. Schreibman. 1997. Humason's animal tissue techniques. The Johns Hopkins University Press, Baltimore, MD.

Rattner, B. A., Gruenau, S. P. and Altland, P. D. 1979. Cross-adaptive effects of cold, hypoxia, or physical training on decompression sickness in mice. Journal of Applied Physiology 47: 412-417.

Snedden, W., Ledez, K., and Manson, H. J. 1996. A new method for the measurement of gas solubility. Journal of Applied Physiology 80: 1371-1378.

Steward, A., Allott P.R., Cowles, A. L., and Mapleson, W. W. 1973. Solubility coefficients for inhaled aneasthetics for water, oil and biological media. British Journal of Anaesthesia 45:282-293.

Struntz, D.J., McLellan ,W.A., Dillaman, R.M., Blum, J.E., Kucklick, J.R., Pabst, D.A. 2004. Blubber development in bottlenose dolphins (Tursiops truncatus). Journal of Morphology 259:7–20.

Swaim, Z. T., Westgate, A. J., Koopman, H. N., Rolland, R. M., and Kraus, S. D. 2009. Metabolism of ingested lipids by North Atlantic right whales. Endangered Species Research 6:259-271.

Tanaka, K., Budd, M. A., Efron, M. L., Isselbacher, K. J. 1966. Isovaleric acidemia: a new genetic defect of leucine metabolism. Proceedings of the National Academy of Sciences 56:236–242.

Wachstein, M. and E. Meisel. 1959. The histochemical demonstration of secretory capillaries in the pancreas with the aid of substrate specific phosphatases. The Journal of Biophysical and Biochemical Cytology 6: 119-120.

Weathersby, P. K., and Homer, L. D. 1980. Solubility of inert gases in biological fluids and tissues: a review. Undersea Biomedical Research 7: 277-296.

Werner, J.A., Schunke, M., and B. Tillmann. 1987. Histochemical visualization of lymphatic capillaries in the rat: A comparison of methods demonstrated at the posterior pharyngeal surface. Archives of histology, Japan 50:505-514.

Wretlind, A. 1957. The toxicity of low-molecular triglycerides. Acta Physiological Scandinavica 40:338–343.

Yamaguchi, K., Mori, M., Kawai, A., Asano, K., Takasugi, T., Umeda, A., Kawashiro, T., and Yokoyama, T. 1993. Effects of pH and SO, on solubility coefficients of inert gases in human whole blood. Journal of Applied Physiology 74: 643-649.